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INTERRELATIONSHIPS BETWEEN WATER AND METABOLISM IN *ARTEMIA SALINA* CYSTS: HYDRATION–DEHYDRATION FROM THE LIQUID AND VAPOUR PHASES

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SUMMARY

Average weights of individual $CaSO_4$-dried cysts (3·64 μg), shells (0·74 μg) and embryos (2·90 μg) of the brine shrimp, *Artemia salina*, were determined. 'Residual water' contents of these preparations were estimated to be 1·58 (cysts), 0·79 (shells), and 1·78 (embryos) in units of g H_2O/100 g dried weight. Cysts hydrated to a similar degree in the liquid or vapour phase of NaCl solutions at 0 °C. Sorption isotherms revealed marked hysteresis for isolated shells, but not for entire cysts or the embryonic component. Adsorption data were plotted according to the Brunauer–Emmett–Teller equation and BET parameters were calculated and discussed. Cyst populations incubated at relative vapour pressures of water of 0·96 and above underwent a loss of dried weight, emerged in the hygrostats, and showed a decrease in viability. The foregoing did not occur at relative vapour pressures of 0·925 and below. The water content required for initiation of these events was estimated to be somewhere in the range of 46 to 72 g H_2O/100 g dried cysts.

INTRODUCTION

One of the major characteristics of living systems is the presence in them of large amounts of intracellular water. However, certain organisms can reversibly, at a given point in their life-cycle, lose essentially all of their intracellular water. I refer here to such things as desiccated spores, seeds, cysts and other developmental stages of species from a diverse array of taxa, and also to adult stages of micrometozoa such as rotifers, tardigrades and nematodes (Keilin, 1959; Grossowicz, Hestrin & Keynan, 1961; Sussman & Halvorson, 1966; Crowe, 1971; Crowe & Clegg, 1973). Relatively few detailed and critical attempts have been made to ascertain the metabolic status of these dried systems, but available evidence strongly suggests that metabolism, in the usual sense of that term, does not occur in organisms containing less than 5% water, by weight (Keilin, 1959; Clegg, 1973). These dried ametabolic, but viable organisms represent a unique state of biological organization (Hinton, 1968) and their unusual properties have been the subject of scientific interest since the beginning of the eighteenth century (see Keilin, 1959). Nevertheless, we know very little about the underlying biochemical and biophysical basis of this condition. Keilin (1959) proposed that the term 'cryptobiosis' replace earlier terminology (abiosis, anabiosis,

suspended animation, etc.) and this term has been widely adopted. Keilin and others (Sussman & Halvorson, 1966; Clegg, 1967; Crowe, 1971) have pointed out that the phenomenon is of interest not only because of its uniqueness, but also because cryptobiotic organisms provide excellent experimental systems in which to explore a wide array of biologically significant problems. One such problem is that of the status and role of water in biological structure and function, a much-debated topic of very considerable importance at the present time (Hazelwood, 1973). It is quite clear from this extensive literature that much remains to be done in this important area of research, and that dried cryptobiotes have rarely if ever been utilized as experimental systems for such studies.

For some time we have been studying the metabolic basis of cryptobiosis in cysts of the brine shrimp, *Artemia salina*, whose suitability for such analyses has been described elsewhere (Finamore & Clegg, 1969). Much is known about the metabolic transitions associated in a general way with hydration and dehydration of these cysts (for references, see Crowe & Clegg, 1973; Dutrieu, 1960; Finamore & Clegg, 1969). However, except for Morris (1971), these investigators either neglected to make measurements on the degree of cyst hydration, or did not relate them in a specific way to the metabolic parameter under study. In fact, no detailed study of water in *Artemia* cysts has been performed.

For these reasons an extensive analysis of the relationships between the level of hydration, the physical state of water, and selected parameters of metabolism in *Artemia* cysts has been initiated.

This paper will describe (1) the processing, characterization, and storage of a very large cyst population to be utilized in all subsequent studies, and (2) initial results on the hydration properties of whole cysts and isolated shells.

MATERIALS AND METHODS

Source of Artemia *cysts*

Cysts collected from salt ponds near Alviso, California, in 1972 were purchased from San Francisco Bay Brand, Inc., Menlo Park, California. In our experience these cysts exhibit much higher viability and more uniformity from year to year than do those collected from the Great Salt Lake, Utah (Longlife Fishfood Products, Harrison, N.J.), and other available commercial sources we have studied. The results presented here have been obtained exclusively with the California strain. Approximately 87 % of this cyst population, after processing, produced viable nauplii when incubated at 25 °C in sea water for 72 h.

Processing and storage of cysts

Commercially obtained cysts are contaminated with sand and other particulate debris. Their shells also contain significant amounts of various entrapped inorganic ions because they originate from a highly saline environment. Therefore they must be thoroughly washed before experiments are performed. The standard washing procedure involves suspending the cysts in 50 times their volume of 0·1 M-NaCl solution for 5 min, then decanting and discarding floating debris, empty shells, and some intact cysts. This procedure is repeated 5 times. The cysts are then floated in saturated NaCl solution and can then be removed from the surface to another container, freeing

them from sand and all debris that sinks in saturated NaCl. This procedure is repeated once. Finally, the cysts are put through five distilled-water washes (100 × the volume of the cysts). It should be emphasized strongly that populations from the Great Salt Lake exhibit considerable lysis during the distilled-water wash, whereas those from the San Francisco area do not. The washed cysts are collected by rapid filtration using filter-paper supports (Schleicher and Schuell, No. 123), given an additional distilled-water wash, and then carefully transferred in small clumps on to absorbent filter paper to air-dry. All these processes are performed at 2–4 °C to suppress metabolic activity, a procedure we have previously shown to be effective (Golub & Clegg, 1968; Clegg & Golub, 1969). After transfer to room temperature for 1 day, the clumps of air-dried cysts are broken up gently by massaging them between filter papers followed by passage through a series of geological sieves to obtain individual cysts. The population used for these studies passed through sieves with 250 μm openings, but were retained by those with openings of 180 μm. The average diameter across the 'rim' of desiccated cysts is about 200 μm. All the cysts used in this series of studies were prepared at one time and then stored over $CaSO_4$ in a nitrogen atmosphere at about −20 °C. One week before use, samples of this population were transferred to room temperature.

Shell preparations

Artemia cysts are composed of a gastrula enclosed within a chitinous shell (see Finamore & Clegg, 1969, for further developmental details). When cysts are incubated aerobically in sea water at 25 °C they resume metabolic activity and the embryos eventually emerge from their shells. Subsequently, the embryos escape from an enclosing envelope as free-swimming nauplius larvae, a process referred to as 'hatching', and leave their shells behind. Empty shells can easily be isolated from cysts and nauplii by flotation in distilled water at 4 °C (the nauplii become inactive and sink along with intact unemerged cysts, while the shells float). Repeated flotations and washes produce preparations of empty shells that are free from all nauplii and emerged embryos, and contain very few intact cysts (three intact cysts were detected in a total of 930 shells taken randomly from such shell preparations). Although most of the shells are intact except for the split made during embryonic emergence, about 5 % of these preparations consist of 'half-shells'. Shells were dried and stored in the same way as the cysts.

Estimation of individual cyst and shell weights

Basically the same procedures were used as previously described (Clegg, 1962). Groups of 10–50 cysts and 50–100 whole shells that had been equilibrated in individual desiccators over $CaSO_4$ were spread on to small pre-weighed squares of transparent 'Scotch' tape that had also been previously equilibrated over $CaSO_4$. After 3 days re-equilibration over $CaSO_4$ they were re-weighed and the average weight per cyst and per shell calculated. Weighings were made on a Mettler–M5 microchemical balance, sensitive to 0·005 mg with a precision of ±0·001 mg.

Hydration–dehydration studies

(*a*) *Aqueous solutions.* Cysts and shells (dried or pre-hydrated) were incubated in NaCl solutions of different concentrations at 0 °C and then assayed for water content

at the appropriate time. This involved suction filtration of the cysts or shells with rapid but thorough washing with distilled water to remove NaCl. The volume of distilled water used in the wash was 100 ml for approximately 20 mg of cysts and the time required for washing was kept constant at 2 min. After suction filtration (15 sec) the cysts were gently blotted and then massaged between filter papers with a rapid but gentle circular motion. If this is done carefully the cyst and shell preparations are rendered into a 'free-flowing powder' in which the individual cysts and shells do not clump together. In effect, this removes water trapped between adhering groups of cysts and on the outermost surface of the shells. This blotting procedure requires about 1 min for 100–200 mg of material.

Bulk water contents of these cyst and shell preparations were estimated by transferring 50–75 mg into small pre-weighed aluminium cups (1 cm deep, 2 cm wide, weighing about 30 mg). After weighing, the individual cups were placed in small separate screw-cap desiccators containing $CaSO_4$ and equilibrated to constant weight (3 days at 25 °C). The use of separate desiccators is necessary for precise work since the time required to weigh a collection of cups in one large desiccator results in appreciable errors due to repeated opening and closing of the desiccator. To reduce 'dead space' and hasten equilibration, small desiccators were used (100 ml volume) and were half-filled with $CaSO_4$. 'Residual water' content of $CaSO_4$-dried preparations was estimated by placing the cysts in an oven for 24 h at 103–105 °C. Unless stated otherwise 'dried' refers to the weight obtained at high temperature. The amount of water was determined by subtraction from the wet weight and the results expressed in grams of water taken up by 100 g of dried cysts. Change in weight as a result of these procedures can be taken as a reliable measure of bulk water content since Morris (1971) has shown that these gravimetric procedures, and use of the Karl Fischer-Reagent analysis for water give comparable results with *Artemia* cysts.

(*b*) *Water vapour sorption.* $CaSO_4$-dried or fully hydrated cysts and shells contained in aluminium cups were placed in screw-cap specimen jars (50 ml) containing solutions of known relative water-vapour pressures and incubated at 25 °C (±0·5 °C). The cups were removed and weighed at suitable intervals to determine equilibrium bulk water content at that a_w (a_w is used to represent both relative vapour pressure of water in vapour phase, and activity of water in solution). Over the range studied the aluminium cups did not adsorb detectable amounts of water vapour.

Cysts and shells, equilibrated as above, were then used either for another sorption cycle, or were re-dried over $CaSO_4$ before being placed in an oven at 103–105 °C for 24 h. The water content after such heating is taken to be zero, and calculations of cyst and shell hydrations are based on this assumption.

The values of a_w (at 25 °C) given below for the various saturated salt solutions used in this study were taken from the data collected by Winston & Bates (1960), and the precautions employed in their preparation and use, as outlined by these authors, were followed.

Salt	a_w	Salt	a_w
LiCl	0·120	$NaKC_4H_4O_6.4H_2O$	0·870
$MgCl_2.6H_2O$	0·325	$ZnSO_4.7H_2O$	0·885
$Na_2Cr_2O_7.H_2O$	0·530	KNO_3	0·925
NaCl	0·755	KH_2PO_4	0·960
KCl	0·850	K_2SO_4	0·975

Table 1. *Average weights of $CaSO_4$-dried cysts, shells and embryos*

Sample no.	Cysts			Shells			Embryos*	Weight of embryos
	No.	μg	μg/cyst	No.	μg	μg/shell	(μg/embryo)	(% cyst wt.)
1	118	436	3·70	115	80	0·70	3·00	81·1
2	96	351	3·66	99	76	0·77	2·89	80·0
3	103	362	3·52	120	88	0·73	2·79	79·3
4	90	330	3·67	113	85	0·75	2·92	79·6
Averages	—	—	3·64	—	—	0·74	2·90	80·0

* Estimated by subtraction of shell from cyst weights.

Table 2. *'Residual water' contents of $CaSO_4$-dried cysts, shells and embryos*

Sample no.	Cysts		Shells		Embryos*	
	g H_2O/100 g dry wt.	μg H_2O/cyst × 10^3	g H_2O/100 g dry wt.	μg H_2O/shell × 10^3	H_2O/100 g dry wt.	μg H_2O/embryo × 10^3
1	1·73	63·0	0·96	7·1	1·93	55·9
2	1·37	49·2	0·58	4·3	1·56	44·9
3	1·49	53·5	0·85	6·3	1·65	47·2
4	1·65	59·2	0·76	5·6	1·88	53·7
5	1·68	60·3	0·81	6·0	1·90	54·4
Averages	1·58	57·0	0·79	5·8	1·78	51·2

* Values for g H_2O/100 g dry wt. embryos were calculated as follows: g H_2O/100 g dry *shells* × 0·2 = g H_2O in the shells of 100 g of dried cysts (Table 1). This number when subtracted from the g H_2O/100 g of dry cysts gives g H_2O/80 g dry embryos which is then converted directly to g H_2O/100 g dry weight of embryos. Residual water per individual embryo was estimated by subtracting shell from cyst hydration.

The water activity of air dried over anhydrous $CaSO_4$ was calculated to be 0·02 using the method of Winston & Bates (1960, p. 235); such $CaSO_4$-dried air contains 5 mg H_2O/l (Bower, 1934).

RESULTS

Standard $CaSO_4$-dried cyst and shell preparations

In order to characterize the cyst population the average weights of individual cysts and shells, dried to equilibrium over $CaSO_4$, were determined (Table 1). These data were then used to estimate, by subtraction, the weight of the dried embryo, which was found to constitute about 80% of the dried cyst weight.

Another parameter of interest is the 'residual water' content of $CaSO_4$-dried preparations. This was measured as described and expressed as g H_2O/100 g of dried cysts or shells (Table 2). These values, and those in Table 1, were then used to calculate the average 'residual hydration' of individual $CaSO_4$-dried cysts, shells, and embryos.

The data in Table 2 indicate that $CaSO_4$-dried cyst populations contain, on the average, a 'residual water' content of 1·58 g H_2O/100 g cysts. However, the method used to estimate this hydration is based on the weight loss occurring when cysts are placed at 103–105 °C for 24 h. The question arises as to whether this weight loss results solely from the removal of water, or whether other volatile components of the

Table 3. *Weight regain by cysts and shells re-equilibrated over $CaSO_4$ following heat treatment of $CaSO_4$-dried preparations*

	Cysts (g/100 g dry wt.)			Shells (g/100 g dry wt.)		
Sample no.	Before heat	After heat	Regain (%)	Before heat	After heat	Regain (%)
1	1·64	0·93	57	0·88	0·79	90
2	1·49	0·62	42	0·63	0·59	94
3	1·54	0·58	38	0·75	0·73	97
Averages	1·56	0·71	46	0·75	0·70	93

Table 4. *Hydration of cysts and shells in NaCl solutions at 0 °C*

	Cysts		Shells	
Incubation medium	g H_2O/100 g dried wt.	Corrected value	g H_2O/100 g dried wt.	Corrected value
'Control'*	6·10	0	23·75	0
0·5 M-NaCl	125·40	119·30	21·99	—
1·0 M-NaCl	94·17	88·07	26·55	—
2·0 M-NaCl	57·85	51·75	19·86	—
3·0 M-NaCl	47·11	41·01	20·10	—
4·0 M-NaCl	38·33	32·23	27·52	—
5·0 M-NaCl	29·11	23·01	20·04	—

* The control values represent dried cysts and shells that were washed and processed in the same fashion as those immersed in the solutions.

cyst are driven off by the treatment. Although this is a very difficult question to answer conclusively, one can evaluate the extent to which the weight loss upon heating is restored when the cysts are subsequently re-incubated over $CaSO_4$. If the loss of weight observed for cysts and shells at high temperature results only from removal of water, then, in the simplest case, one might expect the loss to be restored over $CaSO_4$. Table 3 shows the results of such a study on cysts and shells. The shells regained over 90 % of their weight loss due to heating when re-equilibrated over $CaSO_4$ and it seems safe to conclude that about 0·8 g of water is bound tightly by 100 g of $CaSO_4$-dried shells. The intact cysts, however, regained only about 50 % of their weight when treated in similar fashion, even when kept over $CaSO_4$ for over 4 months.

Hydration in NaCl solutions

The degree to which cysts and shells hydrate when placed in NaCl solutions of decreasing water activity was evaluated and the results are shown in Table 4. These studies were performed at 0 °C to minimize any contributions of cyst metabolism to hydration which have been implicated in a previous study (Clegg, 1964) and will be considered in more detail later in the present study.

Because the preparations had to be washed with distilled water to remove NaCl at the end of the incubation, it was necessary to process $CaSO_4$-dried cysts and shells through a comparable distilled water wash in an attempt to evaluate water uptake due to the washing procedure. Shell hydration cannot be measured by this method since the washing procedure apparently results in maximum hydration, regardless of the

Table 5. *Effects of blotting-time on cyst hydration levels*

Blotting-time (min)	g H_2O/100 g dried cysts* Hydration in 0·5 M-NaCl	Hydration in 4·0 M-NaCl
1	122·7	36·7
2	126·6	34·4
3	124·8	35·8
4	125·0	33·8
5	118·0	31·3
10	108·3	26·2

* Corrected for the washing procedure (see Table 4).

concentration of NaCl solution employed. The cysts, however, exhibit a definite decrease in bulk hydration as a function of increasing NaCl concentration. Because the cysts are known to be essentially impermeable to NaCl (Finamore & Clegg, 1969; unpublished observations) this response is clearly due to the decreased water activity of the solution surrounding the cyst.

A similar series of studies was carried out using cysts that had first been hydrated in 0·05 M-NaCl at 0 °C for 6 days and then transferred to the various NaCl solutions. After 6 days of incubation at 0 °C with occasional mixing, the cysts were processed and their water contents estimated as usual. The results are not given because they are within 5 % of the hydration values for each of the solutions shown in Table 4, a variation considered to be within experimental error. Thus, when suspended in a given NaCl solution the cysts will reach the same hydration level, whether they are dry and undergo hydration, or are hydrated and undergo dehydration.

It should be emphasized that, in order to achieve reproducible results, the washed cysts must be processed to produce a 'free-flowing' preparation. The criterion used for this is the point at which the cysts no longer stick together in small clumps but exist as individuals that can be easily poured from the blotting paper into the weighing cups (see Materials and Methods). At the same time, excessive pressure will rupture the cysts and must be avoided. However, if such precautions are taken the results can be reproduced with precision. Nevertheless, the possibility of error in estimating hydration level due to *removal* of water during blotting had to be evaluated. The cysts were hydrated in 0·5 M-NaCl and 4·0 M-NaCl (0 °C) and were then washed and processed as described. Samples of cysts were taken after increasing times of blotting, quickly weighed, and their water contents determined as usual. Two people were required for this study due to the short times involved. No significant decrease in hydration was evident before about 4–5 min of blotting, and even then the change was very small (Table 5). Since the entire washing–blotting and weighing procedure was routinely accomplished in much less than 5 min per sample it can be concluded that the error involved is trivial.

Hydration from the vapour phase

The results of a study comparing the hydration of cysts from the liquid and vapour phases are given in Table 6. The cysts were incubated at 0 °C, either in aluminium cups over various concentrations of NaCl in sealed hygrostats, or directly within

Table 6. *Hydration of cysts from the liquid and vapour phase of NaCl solutions at* 0 °C

M-NaCl solution	g H_2O/100 g dried cysts		
	Liquid phase*	Vapour phase	Liquid/vapour
0·5	123·24	112·71	1·09
1·0	90·60	92·85	0·98
2·0	52·87	60·60	0·87
3·0	42·31	39·25	1·08
4·0	32·70	31·84	1·03
5·0	28·53	29·22	0·98

* Values for liquid phase hydration have been corrected for the washing procedure (see Table 4).

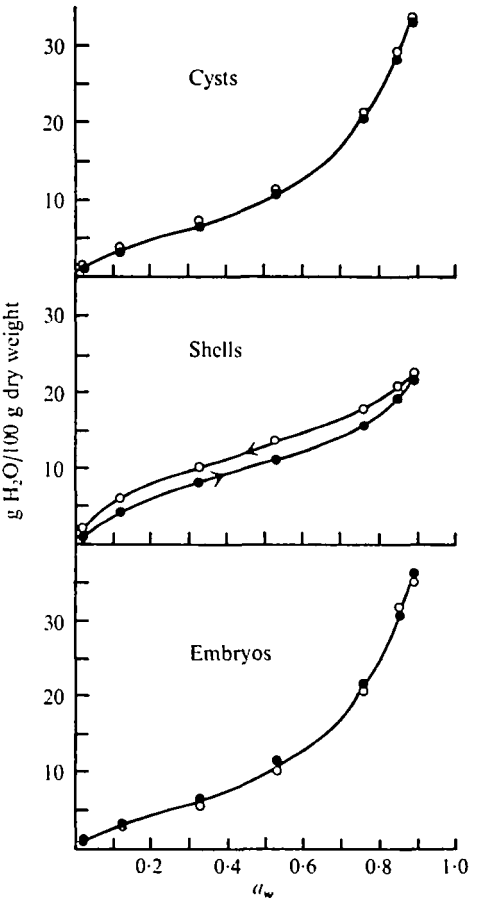

Fig. 1. Sorption isotherms of cysts, shells, and embryos at 25 °C. Each point on the adsorption (●) and desorption (○) cycle represents the average of three separate determinations. a_w represents the relative vapour pressure of water.

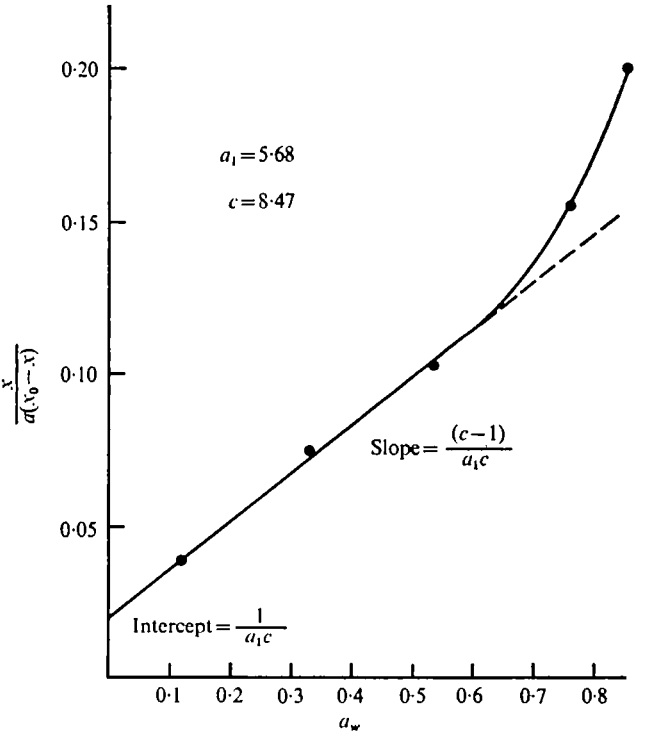

Fig. 2. BET plot using cyst adsorption isotherm data at 25 °C. x = vapour pressure of a given saturated salt solution, x_0 = vapour pressure of pure water, a = hydration of cysts (g H_2O/100 g dried cysts) at a given vapour pressure of water, x. The intercept on the ordinate and the slope of this plot are used, as indicated, to extract the BET constants a_1 and c.

these same solutions. Cysts were incubated for 6 days, since preliminary studies showed that after this period, equilibrium was reached by cysts incubated in the vapour phase at these temperatures. The results showed that the cysts hydrated to similar extents in either case, the differences being quite small and perhaps attributable to experimental variation.

Sorption isotherms for cysts, shells and embryos at 25 °C were obtained by incubating samples of $CaSO_4$-dried cysts and shells at increasing a_w, and then returning them through the reversed series back to $CaSO_4$ (Fig. 1). Values for embryos were calculated from cyst and shell hydration. Very little hysteresis is evident for intact cysts and embryos which is surprising and not at all expected for such an adsorbent, although this is consistent with the results of the earlier studies using NaCl solutions. In contrast, the shells exhibit considerable hysteresis, and of the expected form (i.e. desorption results in higher equilibrium hydration than does adsorption). Isotherms very similar to those shown in Fig. 1 have been obtained by using separate samples of cysts for each a_w studied, thus reducing the time required.

The effect of the previous history of cysts and shells on their hydration properties was evaluated by carrying the cysts through two cycles of adsorption–desorption. The isotherms (not shown) were reproducible in all cases essentially duplicating those shown in Fig. 1.

Fig. 2 depicts a plot of adsorption data for cysts according to the Brunauer–

Table 7. *$CaSO_4$-dried weights of cysts before and after incubation at different water activities* (25 °C)

a_w	$CaSO_4$-dried weights (mg/cup) Before incubation	$CaSO_4$-dried weights (mg/cup) After incubation	Weight change* (Δ μg/mg initial wt. of cysts)
0·530	54·81	54·90	+1·64
0·760	49·93	50·02	+1·80
0·850	58·88	58·93	+0·85
0·870	51·64	51·75	+2·13
0·885	58·29	58·35	+1·03
0·925	57·79	57·88	+1·56
0·960	60·17	59·85	−5·32
0·975	55·15	53·63	−27·56

* (+) indicates an apparent weight gain, (−) an apparent weight loss.

Emmett–Teller (BET) equation (Brunauer, Emmett & Teller, 1938). The data fit the equation well up to water activities of about 0·5 but then deviate from linearity and swing upward. Values of the intercept and slope can then be used, as shown, to calculate the BET parameters a_1 ('monolayer coverage') and c (a constant related to the heat of adsorption of the monolayer). Both of these values are given in Fig. 2 and their interpretation will be considered in the Discussion section. Similar plots were made for shells and embryos and BET constants were calculated: for shells, $a_1 = 5{\cdot}58$ and $c = 17{\cdot}90$; for embryos $a_1 = 5{\cdot}40$ and $c = 8{\cdot}22$.

Studies at higher water activities

Since the dried cysts can be expected to begin metabolic activity at some specified but as yet undetermined hydration level, it was necessary to consider the effects of this on their hydration properties. In preliminary studies it was observed that cysts incubated at high water activities underwent a loss in dry weight during incubation. Such results could be due to metabolic activity of the embryo since previous work has shown (Clegg, 1964) that the cysts will begin to oxidize their endogenous reserves of carbohydrate when a critical hydration level is reached. If sufficient time is allowed for the cyst to degrade appreciable quantities of such substrates at or above this hydration level, then this would be reflected by a net loss in dry weight. To examine this possibility $CaSO_4$-dried cysts were incubated in hygrostats for 4 days at 25 °C, after which they were weighed and then re-dried over $CaSO_4$ and the net change, if any, in $CaSO_4$-dried weight was determined (Table 7). A net loss in dry weight was observed in cysts incubated at an a_w of 0·960 or higher, but not at an a_w of 0·925 or lower. The very small but apparent gain in cyst weight at a_w less than 0·925 seems to be the result of the slight hysteresis exhibited by cysts (Fig. 1).

It must be pointed out that the data shown in Fig. 1 were obtained from cysts incubated at a_w below 0·9. Therefore, those results are due only to water uptake and are not influenced by changes in the dry weight of the cysts during incubation. However, above an a_w of about 0·925 two factors will simultaneously influence the estimates of hydration level: the increase in water, and the decrease in dry mass used to calculate the cyst hydration.

Table 8. *Emergence and viability of cysts incubated at higher water activities* (25 °C)

a_w	Emergence in hydrostats (%)	Viable cysts* (%)
Control ($CaSO_4$)	0	89·3
0·850	0	90·6
0·870	0	88·2
0·885	0	87·2
0·925	0	90·1
0·960	22·1	55·0
0·975	80·6	2·9

* Assayed by removing cysts from hygrostats and incubating them for 72 h in sea water at 25 °C. A cyst is deemed viable only if it produces a swimming nauplius.

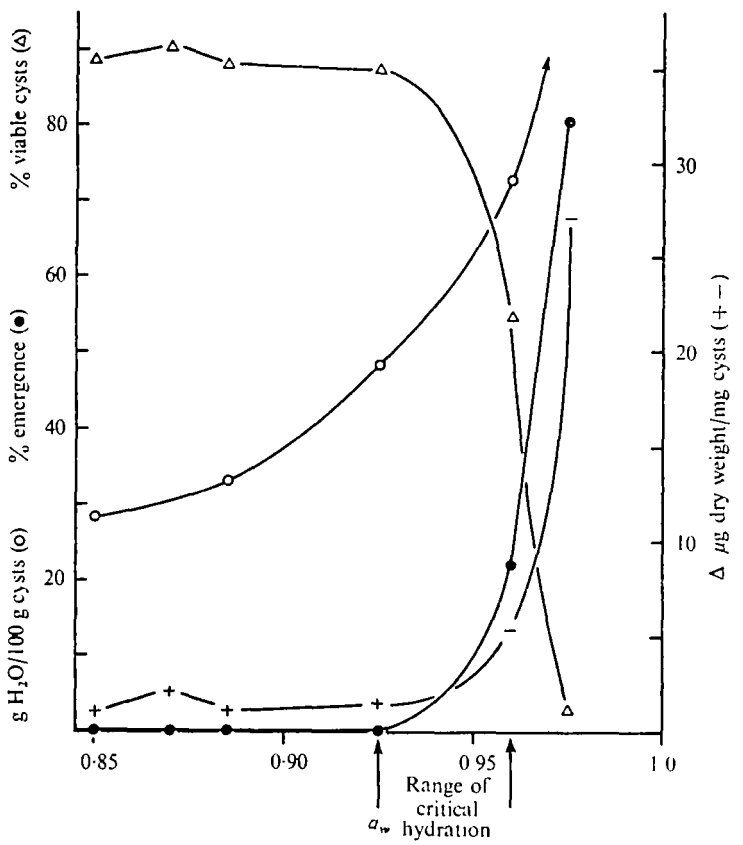

Fig. 3. Summary of cyst hydration (○), emergence in hygrostats (●), viability (△), and change in dry weight (+ or −) as a function of water activity (a_w) at 25 °C. In the case of dry weight changes $a(+)$ indicates an apparent increase, and $a(-)$ an apparent decrease in dry weight during the 4-day incubation period.

Viability and emergence of cysts incubated in the vapour phase

It was noticed that almost all of the cysts incubated at $a_w = 0{\cdot}925$ had taken up sufficient water to swell and become fully spherical, but no emergence was ever observed in these populations. However, some of those incubated at $0{\cdot}960a_w$ had emerged (the E_1 stage of Nakanishi *et al.* 1962). In no case was further development (E_2 or nauplius stages) observed in these populations. Table 8 contains quantitative results on the viability and emergence of cysts that had been incubated at various a_w for 4 days before being removed and assayed for emergence and viability in sea water. These data indicate that only at those hydration levels that result in a decrease in dry mass of the cysts (Table 7) will emergence occur in the hygrostats with concomitant decrease in viability of the population. This can easily be visualized by inspection of Fig. 3, which contains the data of Tables 7 and 8 in addition to the hydration levels (measured as usual) achieved by these preparations during incubation. The 'range of critical hydration level' shown at the bottom of this figure represents the range of cyst water content within which dry weight loss, emergence in the hygrostats, and decrease in viability must begin.

Microscopic examination (× 110 magnification) of cysts incubated at these higher water activities revealed that the surface of the cysts did not appear to contain a film of condensed liquid water. In fact, the surface texture of such cysts appeared similar to those incubated at much lower a_w. Hence, the cysts were not surrounded by a water film of thickness detectable at the magnification used.

DISCUSSION

Standard cyst and shell populations

The average weights of individual $CaSO_4$-dried cysts, shells and embryos (Table 1) differ considerably from my previous measurements of $CaCl_2$-dried individuals (Clegg, 1962). The difference between desiccants cannot account for these results since $CaSO_4$ is a more efficient drying agent than $CaCl_2$ (Winston & Bates, 1960), and both studies utilized cyst populations from the same geographical location. However, the relative contributions of shell and embryo mass to the total cyst weight are very nearly the same in both cases. Perhaps the most obvious explanation is that nutritional differences between the two populations of cyst-producing females resulted in the production of heavier cysts by the 1972 population. These results show that it is necessary to determine weight parameters for each population of cysts obtained from nature, if these values are to be used in calculating the number of cysts in a sample from the total sample mass, and vice versa. Sufficient cysts have been prepared (about 500 g) to provide material for all studies to be done in this series.

'Residual water' in $CaSO_4$-dried cysts and shells

'Residual water' is generally defined as the small amount of water that is not removed from materials dried over strong desiccants and/or at low pressures. Its occurrence and potential importance have often been noted (Meryman, 1960; Neihof, Thompson & Deitz, 1967; Greiff & Rightsel, 1969; Rowe, 1970; Levitt, 1972; Sussman & Douthit, 1973) but details of its nature are poorly understood. In the

case of dried organisms it is likely that much of the 'residual water' is associated with proteins since these substances are known to bind small amounts of water tenaciously (Bull, 1944; Yannas & Tobolsky, 1967). To my knowledge no published information is available to demonstrate that any organism can survive the total removal of its 'residual water'. However, since the method commonly employed to remove residual water (i.e. exposure of the dried organism for relatively long periods to temperatures above 100 °C) can be expected to do a great deal of damage in addition to that caused by the removal of water, the loss of viability in such dried organisms must not, without a great deal of additional evidence, be attributed to the removal of residual water. Furthermore, it is not certain that the only substance removed by high-temperature treatment is 'residual water'; small amounts of a number of compounds in biological systems would perhaps be removed under such conditions. This could account for the observation that intact cysts do not regain their original weight after the heat treatment when returned to $CaSO_4$ (Table 3). Because the shell does regain almost all of its weight under these conditions, it appears that the cellular embryonic mass is the location of such 'volatilized components'. But there could be another explanation for the results in Table 3; heat treatment might cause alterations in the number, conformation, and/or energies of water binding sites within the embryo, thus preventing achievement of the original hydration by regain of water.

Another complexity concerns the question of whether or not all of the 'residual water' is removed by a given time–temperature regime. Thus, although no significant amount of 'residual water' was detected in *Artemia* cysts heated for only 16 h at 103–105 °C in an earlier work (Clegg, 1967), in this study a treatment of 24 h at this temperature did indicate its presence (Table 2).

Levitt (1972), in considering the problems of 'residual water' in resistant plant cells, concludes (p. 328) that although essentially complete desiccation does occur, the question of whether all traces of water can be removed without causing irreversible injury remains to be answered. This also seems to be a fair statement of current understanding of 'residual water' in the cysts of *Artemia*.

Hydration from the liquid phase of NaCl solutions

For a number of reasons to be considered later it is desirable to obtain cysts that are hydrated to known levels but under conditions that reduce metabolism to a standstill. One method used was to measure the water content of cysts that had been incubated in NaCl solutions at 0 °C until equilibrium (Table 4). Two comments are of importance: first, shell hydrations cannot be estimated this way since the washing procedure appears to result in hydration levels equal to those in all NaCl solutions studied; second, the hydration values for intact cysts are subject to error introduced when the NaCl is washed away and the cysts are blotted. This has been controlled to some extent by washing $CaSO_4$-dried cysts in the usual manner, estimating their hydration, and then subtracting this value (control value in Table 4) from the cysts incubated in the solutions. Although this 'control value' is very low compared with the hydration levels achieved by cysts in all the NaCl solutions, the validity of its use rests on the assumption that $CaSO_4$-dried cysts take up an amount of water during the washing procedure that is at least similar to the amounts taken up by cysts at different hydration levels. Some evidence to support this assumption will be presented

in the next section of the discussion. Another potential source of error arises from the blotting procedure used to remove 'surface water' on the outer shell. However, the blotting procedure required to produce 'free-flowing' cyst preparations (see Methods) causes no detectable loss in hydration (Table 5). Errors caused by blotting are, therefore, considered to be negligible.

Comparison of cyst hydration from the liquid and vapour phases

Although the use of NaCl solutions to hydrate cysts to variable water contents is very useful, this approach suffers from two important disadvantages: (1) shell hydrations cannot be performed, (2) it is necessary to remove the incubation solution by washing and then process the cysts before any sort of analysis of the cysts can be carried out. Consequently, attempts to hydrate the cysts and shells from the vapour phase were carried out. The fact that the cysts were found to hydrate to a comparable degree whether immersed in NaCl solutions or suspended in their vapour is important since it strongly supports the assumption that the washing procedure does not introduce unmanageable error into the estimates of hydration of cysts immersed in solutions. Comparable hydrations from vapour and liquid phases can be predicted from the highly sophisticated analysis of Meyers & Sircar (1972*a*, *b*) who related adsorption of liquids and their vapours on solid adsorbents by deriving a thermodynamic consistency test which was then verified experimentally.

A point of some importance concerning shell hydration, and the use of such data to estimate embryo hydration by calculation, is the possibility that empty, isolated shells might exhibit hydration properties that differ from shells surrounding the embryos of intact cysts. Morris & Afzelius (1967) have observed slight differences between the ultrastructure of empty shells isolated after the embryos have emerged, and the shells of intact cysts. However, the authors conclude (p. 256) from their detailed study that 'hydration and development changed the shell structure very little'. It seems probable that these very minor alterations in shell structure are of little, if any, importance in shell hydration. In addition, isolated empty shells clearly present a different geometrical surface to hydrating liquids and vapours than do the shells of intact cysts. Consequently, their hydration might prove to be different in the two morphological states. Although I can think of no empirical way by which this can be critically examined, this probelm is not serious for the present studies since these deal with the equilibrium (or more correctly quasi-equilibrium) hydration states.

Sorption isotherms

The adsorption isotherm obtained for cysts is similar to that obtained by Morris (1971) in spite of very considerable differences in the methods utilized. There seems to be nothing exceptional about the general shape of the adsorption isotherms obtained for shells, cysts and embryos. They resemble those of a wide variety of biological materials, including purified protein powders (Bull, 1944), DNA fibres (Falk, Hartman & Lord, 1962), dehydrated foods (Wolf, Walker & Kapsalis, 1972), red blood cell 'ghosts' (Schneider & Schnieder, 1972), as well as viable dried yeast cells (Koga, Echigo & Nunomura, 1966) and bacterial spores (Neihof, Thompson & Deitz, 1967).

The shells exhibit considerable hysteresis, which might be due to capillarity result-

ng from the porous and vesiculated ultrastructure of the shell as described by Morris & Afzelius (1967) and Anderson *et al.* (1970). What is most unusual, however, is the very slight hysteresis exhibited by the cysts and embryos. Since the embryonic mass clearly swells when sufficient water is taken up it can be expected to exhibit hysteresis. No explanation of such a result will be attempted until further study is made. It is quite apparent, however, that the isotherm of the *entire cyst* is due largely to the properties of the embryonic mass, and is influenced to a much lesser degree by the surrounding shell.

Although more points are needed for accuracy, the adsorption data for cysts appear to give a good fit to the BET equation up to water activities of about 0·5 (Brunauer *et al.* 1938). The question arises as to what interpretation can be applied to the BET parameters (a_1 and c) which have been calculated from this plot. In view of the large number of rigid criteria required under the BET theory, and the structural complexity of the cyst, it seems quite evident that the value of a_1 cannot be interpreted as the amount of water involved in the covering of the adsorbing surface by a single layer of water molecules (the so-called monolayer coverage). Nevertheless, values of a_1 have been obtained for a number of biological materials and the value for *Artemia* cysts are interesting in a comparative sense. In units of g H_2O adsorbed by 100 g dried material, values of a_1 for proteins and polysaccharides are usually in the range of 5–12 whereas values for nucleic acids are perhaps twice as great (see Schneider & Schneider, 1972; Falk *et al.* 1962). The value of a_1 for *Artemia* cysts (5·7) is very close to the calculated value for red blood cell ghosts (Schneider & Schneider, 1972) and appears to be similar to that of vegetative yeast cells based on the sorption data of Koga *et al.* (1966).

It is more informative, however, to compare the area that would be covered by this amount of water (the specific surface area) with the calculated external surface area of the cysts. If the commonly used value of 10·5 Å^2 for the area covered by one water molecule is assumed then the specific surface area (S) can be calculated as follows (Brunauer, 1945):

$$S = \frac{N\theta a_1}{v_0},$$

where N is Avogadro's number, θ is the area covered by a water molecule, a_1 is 'monolayer coverage' (0·0568 gH_2O/g cysts), and v_0 the molar volume of water vapour at standard temperature and pressure. The calculated value for the area covered by a_1 is 160 m^2/g of cysts. This can now be compared to the geometric external surface area of *Artemia* cysts: 0·035 m^2/g cysts (using an average cyst diameter of 0·2 mm, obtained from unpublished observations, and an average weight of 3·6 μg per cyst, from Table 1). The cyst can be taken as a sphere in this calculation, even when dry, since the hydrated cyst is spherical and the surface area of the shell does not change appreciably as a function of its hydration. Even if we allow for surface roughness, it is evident that these two values differ by a factor of the order of 10^4, suggesting rather strongly that 'monolayer hydration' of *Artemia* cysts involves, to a very great degree, hydration of sites within the cells of the embryo and is not limited to the non-cellular shell. The extent to which this is the case can be evaluated from the a_1 value obtained from BET plots of adsorption data on shells, and its use to calculate specific surface

area of shells. The 'monolayer coverage' for the shells contained within 1 g of cyst[redacted] is thus estimated to be 0·0112 g H_2O, which gives a specific surface area, for this amount of water, of 32 m^2/g cysts. Consequently, it appears that the 'monolayer coverage' in the embryonic mass amounts to about 128 m^2/g cysts. By further study it is hoped that a more specific meaning can be attached to the value of a_1 in *Artemia* cysts.

The BET constant, c, is supposed to be related to the heat of water vapour adsorption, but the same restrictions in interpretations of a_1 also apply in this case. Interestingly, however, the value of c for the cysts (8·1) is very close to that obtained by Schneider & Schneider (1972) for red blood cell ghosts (7–8·3 at 20 °C), and not far from the range of c often encountered in a variety of other biological materials.

Hydration at higher water activities and 25 °C

It has been shown previously that cysts incubated at 25 °C in NaCl solutions up to concentrations of about 1 molal adjust their metabolism (Clegg, 1964). Apparently this adjustment occurred in such a way as to compensate for low environmental water activities and, thereby, to enhance cyst hydration at that water activity, though no measurements were carried out on the levels of cyst hydration. In that study, evidence was also presented that cysts incubated in solutions of 2 M-NaCl were unable to initiate sufficient metabolism to compensate in this fashion. These data are of considerable importance to the present study.

Since the a_w of a 2 M-NaCl solution at 25 °C is about 0·93 and that of a 1 M-NaCl solution is about 0·97 (Pearce & Nelson, 1932), it follows that such metabolism, and predictably therefore emergence, should begin at some a_w within this range. This should be the case whether hydration occurs via vapour or liquid phases since cyst hydration levels have been shown to be similar from either phase (Table 6). Results of studies in which hydration was carried out at 25 °C from the vapour phase in the present study (Tables 7, 8; Fig. 3) are in accord with these observations. Thus, the cysts exhibited no change in $CaSO_4$-dry mass, did not emerge in the hygrostats, and showed no decrease in viability levels (compared to the dry controls) up to water activities of 0·925. However, at a_w = 0·96 (comparable to the water activity of a 1·2 M-NaCl solution) all of these three events took place, increasing in degree with higher environmental water activities. Consequently, it is possible to estimate a range of cyst hydration within which a critical point is reached at which the cysts can initiate metabolic activity sufficient to bring about emergence. This has been referred to as the 'range of critical hydration' and occurs between 46 and 72 g H_2O/100 g of dried cysts (Fig. 3). The nature of this metabolic activity and the observation that the viability of the cyst populations decreases at or above the 'critical hydration level' will be investigated in subsequent studies. However, it should be stressed now that the isotherm data shown in Figs. 1 and 2 are free from such complications since they were obtained at water activities well below those required to produce the 'critical hydration level' in the cysts.

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REFERENCES

ANDERSON, E., LOCHHEAD, J. H., LOCHHEAD, M. S. & HUEBNER, E. (1970). The origin and structure of the tertiary envelope in thick-shelled eggs of the brine shrimp, *Artemia*. *J. Ultrastruc. Res.* **32**, 497–525.

BOWER, T. (1934). Efficiency of drying agents. *Bureau Stand. J. Res.* **12**, 241.

BRUNAUER, S. (1945). *Physics of Adsorption of Gases and Vapors*, p. 287. Oxford University Press.

BRUNAUER, S., EMMETT, P. H. & TELLER, E. (1938). Adsorption of gases in multimolecular layers. *J. Am. Chem. Soc.* **60**, 309–21.

BULL, H. B. (1944). Adsorption of water vapor by proteins. *J. Am. Chem. Soc.* **66**, 1499–507.

CLEGG, J. S. (1962). Free glycerol in dormant cysts of the brine shrimp, *Artemia salina*, and its disappearance during development. *Biol. Bull. mar. biol. Lab., Woods Hole* **123**, 295–301.

CLEGG, J. S. (1964). The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. *J. exp. Biol.* **41**, 879–92.

CLEGG, J. S. (1967). Metabolic studies of cryptobiosis in encysted embryos of *Artemia salina*. *Comp. Biochem. Physiol.* **20**, 801–9.

CLEGG, J. S. (1973). Do dried cryptobiotes have a metabolism? In *Anhydrobiosis* (ed. J. H. Crowe and J. S. Clegg), pp. 141–6. Stroudsburg, Pa.: Dowden, Hutchinson and Ross.

CLEGG, J. S. & GOLUB, A. L. (1969). Protein synthesis in *Artemia salina* embryos. II. Resumption of RNA and protein synthesis upon cessation of dormancy. *Devl Biol.* **19**, 178–200.

CROWE, J. H. (1971). Anhydrobiosis: an unresolved problem. *Am. Nat.* **105**, 563–74.

CROWE, J. H. & CLEGG, J. S. (ed.) (1973). *Anhydrobiosis*. Stroudsburg, Pa.: Dowden, Hutchinson and Ross.

DUTRIEU, J. (1960). Observations biochimiques et physiologiques sur le développement d'*Artemia salina* Leach. *Archs Zool. exp. gén.* **99**, 1–133.

FALK, M., HARTMAN, K. A. & LORD, R. C. (1962). Hydration of DNA. I. A gravimetric study. *J. Am. Chem. Soc.* **84**, 3843–6.

FINAMORE, F. J. & CLEGG, J. S. (1969). Biochemical aspects of morphogenesis in the brine shrimp, *Artemia salina*. In *The Cell Cycle: Gene–Enzyme Interactions* (ed. G. M. Padilla, G. L. Whitson and I. L. Cameron), pp. 249–78. New York: Academic Press.

GOLUB, A. & CLEGG, J. S. (1968). Protein synthesis in *Artemia salina* embryos. I. Studies on polyribosomes. *Devl Biol.* **17**, 644–56.

GREIFF, D. & RIGHTSEL, W. A. (1969). Stabilities of dried suspensions of influenza virus sealed in a vacuum or under different gases. *Appl. Microbiol.* **17**, 830–5.

GROSSOWICZ, N., HESTRIN, S. & KEYNAN, A. (ed.) (1961). *Cryptobiotic Stages in Biological Systems*. New York: Elsevier.

HAZELWOOD, C. F. (ed.) (1973). Physico-chemical state of ions and water in living tissues and model systems. *Ann. N.Y. Acad. Sci.* **204**, 1–631.

HINTON, H. E. (1968). Reversible suspension of metabolism and the origin of life. *Proc. Roy. Soc. B* **171**, 43–57.

KEILIN, D. (1959). The problem of anabiosis or latent life: history and current concepts. *Proc. R. Soc. Lond.* B **150**, 149–91.

KOGA, S., ECHIGO, A. & NUNOMURA, K. (1966). Physical properties of cell water in partially dried *Saccharomyces cerevisiae*. *Biophys. J.* **6**, 665–74.

LEVITT, J. (1972). *Responses of Plants to Environmental Stresses*. New York: Academic Press.

MERYMAN, H. T. (1960). Drying of living mammalian cells. *Ann. N.Y. Acad. Sci.* **85**, 729–34.

MEYERS, A. L. & SIRCAR, S. (1972*a*). A thermodynamic consistency test for adsorption of liquids and vapors on solids. *J. Phys. Chem.* **76**, 3412–14.

MEYERS, A. L. & SIRCAR, S. (1972*b*). Analogy between adsorption from liquids and adsorption from vapors. *J. Phys. Chem.* **76**, 3415–19.

MORRIS, J. E. (1971). Hydration, its reversibility, and the beginning of development in the brine shrimp, *Artemia salina*. *Comp. Biochem. Physiol.* **39***A*, 843–57.

MORRIS, J. E. & AFZELIUS, B. A. (1967). The structure of the shell and outer membranes in encysted *Artemia salina* embryos during cryptobiosis and development. *J. Ultrastruc. Res.* **20**, 244–59.

NAKANISHI, Y. H., IWASAKI, T., OKIGAKI, T. & KATO, H. (1962). Cytological studies of *Artemia salina*. I. Embryonic development without cell multiplication after the blastula stage. *Annotnes zool. jap.* **35**, 223–8.

NEIHOF, R., THOMPSON, J. K. & DEITZ, V. R. (1967). Sorption of water vapor and nitrogen gas by bacterial spores. *Nature, Lond.* **216**, 1304–6.

PEARCE, J. W. & NELSON, A. F. (1932). The vapor pressure of aqueous solutions of lithium nitrate and the activity coefficients of some alkali salts in solutions of high concentration at 25 °C. *J. Am. Chem. Soc.* **54**, 3544–55.

ROWE, T. W. G. (1970). Freeze-drying of biological materials: some physical and engineering aspects. In *Current Trends in Cryobiology* (ed. A. U. Smith), pp. 61–139. New York: Plenum Press.

SCHNEIDER, M. J. T. & SCHNEIDER, A. S. (1972). Water in biological membranes: adsorption isotherms and circular dichroism as a function of hydration. *J. Membrane Biol.* **9**, 127–40.

SUSSMAN, A. S. & DOUTHIT, H. A. (1973). Dormancy in microbial spores. *A. Rev. Pl. Physiol.* **24**, 311–52.

SUSSMAN, A. S. & HALVORSON, H. O. (1966). *Spores: Their Dormancy and Germination*. New York: Harper and Row.

WINSTON, P. W. & BATES, D. H. (1960). Saturated solutions for the control of humidity in biological research. *Ecology* **41**, 232–7.

WOLF, M., WALKER, J. E. & KAPSALIS, J. G. (1972). Water vapor sorption hysteresis in dehydrated foods. *Agr. Food Chem.* **20**, 1073–7.

YANNAS, I. V. & TOBOLSKY, A. V. (1967). Cross-linking of gelatine by dehydration. *Nature, Lond.* **215**, 509–10.